

## METHODS AND COMPOSITIONS FOR DETECTING TELOMERASE ACTIVITY

### CROSS REFERENCE TO RELATED APPLICATION(S)

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/425,620, filed November 12, 2002, the disclosure of which is hereby incorporated by reference.

### 5 TECHNICAL FIELD

The present invention generally relates to medical diagnostic and prognostic technology. In particular, the present invention relates to a method for the detection of telomerase activity.

### BACKGROUND

10 Telomerase is an enzyme that synthesizes telomeres on chromosome ends. Telomeres are DNA sequences found at the ends of eukaryotic chromosomes which maintain the fidelity of genetic information during replication. Under normal circumstances, telomeres become shorter and shorter with each cycle of cell division. A sufficiently short telomere is believed to signal the cells to stop dividing.

15 Telomerase belongs to a class of enzymes known as reverse transcriptases that use RNA as a template for creating DNA. Telomerase contains both RNA and protein components. The RNA portion of the enzyme binds to the DNA in the telomere while the protein component lures DNA subunits into the region and attaches them to the end of the chromosome. Telomerase then elongates the G-rich strand of chromosomal termini by  
20 adding telomeric repeats. This elongation occurs by reverse transcription of a part of the telomerase RNA component, which contains a sequence complementary to the telomere repeat. Following telomerase-catalyzed extension of the G-rich strand, the complementary DNA strand of the telomere is presumably replicated by more conventional means. In the case of eukaryotic organisms, telomerases are composed of  
25 an accumulation of repeated defined nucleotide sequences (repeats) which, for example, contain the sequence TTAGGG in humans.

Telomerase activity is not detectable in normal tissues except germline cells. Germline cells, whose chromosomal ends must be maintained through repeated rounds of DNA replication, do not decrease their telomere length with time, presumably due to the  
30 activity of telomerase. Stem cells of renewing tissues express very low levels of

telomerase and their telomeres shorten with multiple cell divisions. Telomerase activity is occasionally detected in tissues adjacent to tumors possibly reflecting the presence of occult micrometastases.

Telomerase is believed to have a role in the process of cell senescence. The repression of telomerase activity in somatic cells is likely to be important in controlling the number of times they divide. Indeed, the length of telomeres in primary fibroblasts correlates well with the number of divisions these cells can undergo before they senescence. The loss of telomeric DNA may signal to the cell the end of its replicative potential, as part of an overall mechanism by which multicellular organisms limit the proliferation of their cells.

Due to its role in controlling replication, telomerase has also recently been implicated in oncogenesis. Telomerase activity has been detected in most tumor cells. It has been suggested that telomerase is responsible for the unchecked growth of human cancer cells. Unlike normal cells, in cancer cells telomerase appears to grant the cell immortality by maintaining telomere length so that the cell never receives a signal to stop dividing. The telomerase enzyme is an ideal target for chemotherapy because this enzyme is active in about 90 percent of human tumors, but inactive in most normal cells. Pharmaceutical companies have screened thousands of compounds to find agents capable of blocking telomerase.

A method termed as telomeric repeat amplification protocol (TRAP) has been developed to measure telomerase activity. TRAP is based on the *in vitro* detection of the enzyme activity. Briefly, a synthetic oligonucleotide derived from the telomere sequence is used as a substrate. This substrate is elongated by the telomerase in a test sample and the elongation product is then amplified and quantified. Detailed description of the TRAP methods can be found in, for example, U.S. Patent No. 5,891,639 to Harley *et al.* (hereinafter Harley) and U.S. Patent No. 6,221,584 to Emrich *et al.* (hereinafter Emrich). Recently, a number of research groups have reported modified TRAP methods using real-time polymerase chain reaction (PCR) technology (See *e.g.*, Hou *et al.*, Clin. Chem. 47:519-524, 2001; Elmore *et al.*, Diagn. Mol. Pathol., 11,177-185, 2002; and Wege *et al.*, Nucleic Acids Res., 31:E3-3, 2003). Specifically, real-time PCR technology has been employed to provide a faster and more sensitive quantification of the elongation product of telomerase.

The current TRAPs typically include multiple incubation steps and transfer of sample from one tube to another after each incubation step. The transferring process is

time consuming and prone to contamination and operation error (e.g., adding samples to a wrong tube or well). The current TRAPs usually start with a cell or tissue extract. Since telomerases, which contain both RNA and protein components, are subjected to the digestion of proteases and RNases in the extract, protease inhibitors and/or RNase inhibitors are often needed to prevent the degradation of the telomerases. Addition of protease inhibitors and/or RNase inhibitors to the extract increase the cost of the analysis. Thus, a need exists for an telomerase assay that is more flexible and can be performed easily and quickly at a low cost.

### SUMMARY

A method for determining telomerase activity using primer extension followed with real-time PCR quantification is disclosed. The method provides a rapid, sensitive and accurate measurement for telomerase activity in a biological sample.

In one embodiment, the method includes the steps of: (1) adding the biological sample to a reaction tube containing a first reaction mixture having a first primer and nucleoside triphosphates, a second reaction mixture having a second primer and a DNA polymerase, and a wax layer that separates the first reaction mixture from the second reaction mixture; (2) incubating the biological sample with the first reaction mixture under conditions suitable for a telomerase to produce an extension product from the first primer; (3) admixing the extension product with the second reaction mixture; (4) amplifying the extension product using real-time PCR under conditions that allow the detection of telomerase activity from a single 293T cell; and (5) quantifying the amplified extension product using a control template that is amplified under the conditions in step (4). The single tube design of the embodiment simplifies the experimental procedure and reduces experimental error.

In another embodiment, the detection method includes the steps of: (1) introducing into a sample cell a first primer and nucleoside triphosphates; (2) incubating the sample cell under conditions suitable for a telomerase to produce an extension product from the first primer inside the cell (*in situ* primer extension); (3) amplifying the extension product using real-time PCR; and (4) quantifying the amplified extension product using a control template that is amplified under the conditions in step (3). In this embodiment, the extension product is produced within an intact sample cell and can be preserved under appropriate conditions for an extended time before the completion of the quantification step.

Also disclosed is a reagent kit for carrying out the method and for diagnosing telomerase-related diseases. In one embodiment, the reagent kit includes (1) reaction tubes that contain a first reaction mixture having a first primer and nucleoside triphosphates, a second reaction mixture having a second primer and a DNA polymerase, and a wax layer that separates the first reaction mixture from the second reaction mixture; and (2) control tubes or wells that contain a first reaction mixture having a first primer and nucleoside triphosphates, a second reaction mixture having a second primer, a DNA polymerase, and a control template, and a wax layer that separates the first reaction mixture from the second reaction mixture.

## 10 **BRIEF DESCRIPTION OF DRAWINGS**

The detailed description will refer to the following drawings, in which like numerals refer to like elements, and in which:

Figure 1 is a schematic drawing depicting an embodiment of the method for detecting telomerase activity.

15 Figure 2 is a schematic drawing depicting an additional extension step in the method for detecting telomerase activity.

Figure 3 is a schematic drawing depicting another embodiment of the method for detecting telomerase activity.

20 Figures 4A and 4B are a fluorescence/PCR cycle plot and a threshold cycle/cell number plot, respectively, that demonstrate the sensitivity of the detection method.

Figures 5A and 5B are a fluorescence/PCR cycle plot and a threshold cycle/template molecule number plot, respectively, generated by real-time PCR using a control template.

25 Figure 6 is a plot showing linear regression between sample cell number and threshold cycle or between template molecule number and threshold cycle.

Figure 7 is a bar plot showing telomerase activity in various cell lines and tissues

Figure 8 is a fluorescence/PCR cycle plot showing the detection of telomerase activity in cells subjected to *in situ* primer extension after syringe treatment.

30 Figure 9 is a fluorescence/PCR cycle plot showing the detection of telomerase activity in cells subjected to *in situ* primer extension after calcium phosphate precipitation.

**DETAILED DESCRIPTION**

Figure 1 shows a method 100 for detecting and quantifying telomerase activity in a biological sample. First, the biological sample is added to a reaction tube that contains a first reaction mixture having a first primer and nucleoside triphosphates and a second reaction mixture having a second primer and a DNA polymerase (step 102). The first reaction mixture is separated from the second reaction mixture by a layer of wax that melts at high temperatures. The biological sample is mixed with the first reaction mixture, which occupies the top portion of the reaction tube, and incubated under conditions suitable for a telomerase to produce an extension product from the first primer (step 104). The extension product is then mixed with the second reaction mixture by melting the wax layer (step 108), and is amplified by real-time PCR (step 110). The unextended first primer in the first reaction mixture and the second primer in the second reaction mixture form the primer pair for PCR amplification. The telomerase activity in the biological sample is then quantified by comparing the amount of PCR product in the reaction tube to the amount of PCR product in control tubes having known amounts of a control template (step 112). In this embodiment, the experimental conditions for the first primer extension and the PCR reaction have been optimized to allow the detection of telomerase activity from a single 293T cell.

The biological sample includes tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and biological fluids present within a subject. The biological sample also includes primary cells, transformed cells, and any other cultured cells. Since the method of the present invention detects the activity of telomerase, a RNase sensitive ribonucleoprotein, and not merely the presence of the RNA or protein components of telomerase, the method requires enzymatically active cell or tissue samples. In one embodiment, the biological sample is a tissue sample isolated by conventional means from a subject, *e.g.*, a biopsy. Preferably, the biological sample is a cell or tissue extract, in particular an extract from human cells or tissues. The extract may be produced by repeated thawing/freezing of cells, by homogenizing cells or tissues, or by lysing cells or tissues in a lysis buffer containing a non-ionic or/and zwitterionic detergent. Examples of the non-ionic detergent include, but are not limited to, Tween 20, Triton X-100, Triton X-114, polydocanol (Thesit), NP-40, n-octylglucoside, n-dodecylglucoside, n-dodecyl-beta-D-maltoside, octanoyl-N-methylglucamide (MEGA-8), decanoyl-N-methylglucamide (MEGA-10), and isotridecyl-poly(ethyleneglycolether)<sub>n</sub>. Examples of the zwitterionic detergents include, but are not limited to, CHAPS (3-[(3-

cholamidopropyl)dimethylammonio]-1-propane-sulfonate), CHAPSO (3-[(3-cholamidopropyl)dimethyl-ammonio]-2-hydroxy-1-propane-sulfonate), N-dodecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate, and digitonin. The amount of detergent in the lysis buffer may vary from about 0.1% to about 2% by weight. In one embodiment, the lysis buffer contains about 0.5% detergent by weight.

Since telomerase contains both RNA and protein components, protease and/or RNase inhibitors may be added to the extract to prevent the destruction of telomerases in the extract by other cellular proteases. Examples of the protease inhibitors include, but are not limited to, amastain, 4-amidinophenylmethanesulfonyl fluoride (AMPSF), antipain, aprotinin, bestatin, chymostatin, cystatin, 3,4-dichloroisocoumarin, ebelactone A and B, elastatinal, ethylenediamine tetra-acetic acid (EDTA), ethylene glycol tetra acetic acid (EGTA), leupeptin, pepstatin A, phenylmethyl sulfonyl fluoride (PMSF), phosphoramidon, tosyl lysyl chloromethylketone (TLCK), tosyl phenylalanyl chloromethylketone (TPCK), and trypsin inhibitors. Examples of RNase inhibitors include, but are not limited to, pancreatic-type RNase inhibitors, human placenta RNase inhibitors, and diethyl pyrocarbonate (DEPC).

The reaction tube can be a container of any shape or size that fits the requirement of a particular application of the method. Typically, the reaction tube is a PCR tube or a PCR well as is well-known to one skilled in the art.

The first primer in the first reaction mix is an oligodeoxyribonucleotide suitable as a telomerase substrate. The first primer serves two functions: it serves as a substrate for the telomerase to produce an extension product, and it also serves as a primer in the subsequent PCR reaction. In one embodiment, the length of the first primer is 10-60 nucleotides. In another embodiment, the length of the first primer is 12-30 nucleotides. Preferably, the first primer, which serves as the telomerase substrate, does not contain a complete telomeric repeat sequence of the particular telomerase that will use the first primer as a substrate. For example, human telomerase adds telomeric repeats of sequence 5'-TTAGGG-3' (SEQ ID NO:1). Accordingly, if one is using the present method to assay for human telomerase activity, the telomerase substrate should be a human telomerase substrate lacking the complete repeat sequence 5'-TTAGGG-3'. The reason is that telomerase can extend the telomerase substrate only by the addition of telomeric repeats. Therefore, the second primer, which is to form a primer pair with the first primer in the PCR reaction, will necessarily comprise a sequence complementary to a telomeric repeat. If the first primer (*i.e.*, the telomerase substrate) employed in the telomerase extension

reaction comprises a complete telomeric repeat, then the second primer employed in the PCR reaction could hybridize readily to the unextended first primer and form primer-dimers that will potentially lead to negative PCR results.

The nucleoside triphosphates in the reaction mix include, but are not limited to, deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxyuridine triphosphate (dUTP), deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP). In one embodiment, the reaction mixture contains dATP, dGTP, dCTP and one of dUTP and dTTP, in equal molar ratio. The nucleoside triphosphates are designated collectively as dNTPs.

In addition to the first primer and the nucleoside triphosphates, the first reaction mixture may also contain a buffer system to maintain an optimal pH for the primer extension reaction for the telomerase. Examples of the buffer systems include, but are not limited to, phosphate buffer system, citrate buffer system, borate buffer system, Tris-(hydroxymethyl)aminomethane, 3-[(3-cholamidopropyl)dimethylammoniol]-1-propane sulfonate (CHAPS), N-[2-hydroxyethyl]piperazine-N'-2-[ethanesulfaonic acid] (HEPES), and 3-[N-morpholino]propanesulfonic acid (MOPS).

The conditions suitable for a telomerase to produce an extension product from the first primer are well-known in the art. Typically, the telomer extension reaction is performed at about 20-30°C for about 10-60 min, preferably at about 25°C or about 30°C for about 15-30 min, and most preferably at about 25°C for about 20 min.

The extension product of the step 104 may be subjected to additional template-independent elongation (step 106). This elongation is preferably achieved by means of an enzymatic reaction *e.g.* by attaching nucleotides using terminal transferase or by ligation of short DNA fragments using DNA ligase. In one embodiment, a polyA tail is added to the 3' end of the extension product by terminal transferase. In another embodiment, a short DNA oligomer of 10-20 nucleotides is ligated to the 3' end of the extension product. These modifications generate a unique sequence for the second primer and thus allow the inclusion of the complete telomeric repeat sequence in the first primer. As shown in Figure 2, a first primer containing a human telomeric repeat TTAGGG is used as a human telomerase substrate. After the telomerase-mediated extension, the extension product will have a 3' sequence of (TTAGGG)<sub>n</sub>TTAGGGTTAGGG-3', where n is an integer that is equal to or is greater than zero. After the additional template-independent elongation that adds a polyA tail of at least 10 nucleotides to the 3' end of the extension product, the elongated extension product will have a 3' sequence of (TTAGGG)<sub>n</sub>TTAGGGTTAGGGAAAAAAAAAAAAA<sub>m</sub>-3',

where  $n$  is an integer that is equal to or is greater than zero. The second primer can then be designed to have a sequence complementary to the junction of the telomeric repeat sequence and the polyA sequence at the 3'-end of the elongated extension product. As shown in Figure 2, a second primer complementary to the junction of the telomeric repeats and the additional nucleotides should be able to distinguish the unextended first primer from the extended product, so long as the first primer does not have a complete telomeric repeat sequence at its 3'-end.

In the absence of the additional elongation step 106, the second primer in the second reaction mixture typically contains multiple imperfect telomeric repeat sequences and at least one perfect telomeric repeat sequence to minimize the formation of non-specific PCR products such as primer-dimer.

The DNA polymerase in the second reaction mixture can be any DNA polymerase suitable for standard PCR conditions. Such enzymes are well-known to one skilled in the art. In an embodiment, the second reaction mixture also contains a magnesium salt that provides the optimal magnesium for the PCR amplification of the extension product. In another embodiment, the second reaction mixture also contains the first primer, or dNTP, or both.

The wax layer that separates the first reaction mixture from the second reaction mixture should have a melting temperature within the range of about 50-90°C, and preferably within the range of about 60-80°C. In an embodiment, a trace amount of a dye may be added to one of the first reaction mixture and the second reaction mixture to monitor possible leakage through the wax layer prior to the PCR amplification. In another embodiment, the second reaction mixture is premixed with the wax and are confined within the wax layer when the wax solidifies. The contents of the second reaction mixture are released when the wax layer is melted at a higher temperature.

The extension product (with or without additional elongation) is quantified by the real-time PCR amplification. As is known to one skilled in the art, PCR amplification is typically achieved by adding a thermostable enzyme and a pair of primers to a reaction mixture containing a template and going through multiple thermocycles. In method 100, the unextended first primer in the first reaction mixture serves as the 5' PCR primer and the second primer in the second reaction mixture serves as the 3' PCR primer.

The PCR product in a real-time PCR reaction can be detected using fluorescence resonance energy transfer (FRET) technology. The accumulation of a specific PCR



product can be measured by comparing the linear portion of each amplification to a standard curve generated using a known template.

In one embodiment, the PCR reaction is carried out normally but with the addition of a fluorescently labeled probe oligonucleotide that binds to a sequence between the two flanking PCR primers. The method relies on the 5' exonuclease activity of Taq polymerase to cleave a fluorescently labeled nucleotide from the 5' end of the probe. The probe oligonucleotide also has a fluorescent quencher at the 3' end that suppresses the overall fluorescence, therefore, when the 5' labeled nucleotide is removed the quenching effect is lost because the distance between the two fluorophores is too great to interfere with each other. Each cycle produces further increases in fluorescence allowing the whole PCR reaction to be followed in real time. The amount of template DNA present in the reaction can be calculated by comparing the linear part of the exponential amplification with a standard curve. Examples of such detection system include, but are not limited to, TaqMan<sup>®</sup> system (Applied Biosystems, Foster City, CA).

In another embodiment, the PCR product is detected by using single-labeled fluorogenic primers, such as the LUX<sup>®</sup> primers (Invitrogen, Carlsbad, CA) and Amplifluor RP<sup>®</sup> primers (Chemicon, Temecula, CA). The primers produce increased amount of fluorescence emission when the fluorogenic primer is incorporated into double-stranded PCR product. The amount of the PCR product then be determined based on the fluorescence produced during the amplification step of each PCR cycle in the closed reaction tube.

In yet another embodiment, the PCR product is detected using a fluorescent dye that binds preferentially to double-stranded DNA. The dye, such as SYBR<sup>®</sup> Green, can thus accurately quantitate the amount of double-stranded product made in the presence of single-stranded oligonucleotide primers.

The telomerase activity is quantified by comparing the increase of fluorescence in the reaction tube to the increase of fluorescence in a control tube that contains a known amount of a control template (step 110). Typically, real-time PCR is performed to generate a standard curve using a set of control tubes or wells that contain different dilutions of the control template. The extension product in the test tubes or wells are then amplified under identical PCR conditions and the telomerase activity in the test tubes or wells are quantified based on the standard curve. Telomerase activity is usually expressed as the amount of telomeric repeats synthesized within a certain period of time.

AATCCGTCGAGCAGAGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG  
TTAGGGTTAGGGTTAG-3' (SEQ ID NO:2).

Figure 3 shows another method 300 for the detection of telomerase activity. In this embodiment, the primer extension step is performed within an intact cell. Specifically, the first primer and nucleoside triphosphates are introduced into an intact sample cell (step 302); the sample cell is then incubated under conditions suitable for the telomerase within the cell to produce an extension product from the first primer while maintaining the integrity of the cellular structure (step 304). The step 304 is also referred to as the “*in situ* primer extension step” because the extension product is generated inside the sample cell. The extension product is then amplified and quantified using real-time PCR and a control template (step 308). In one embodiment, the sample cell is mixed with the second reaction mixture and subjected to PCR amplification. In another embodiment, the sample cell is lysed in a lysis buffer and the lysate is used in the real-time PCR reaction.

Alternatively, the sample cell may be stored after the completion of the telomerase-mediated primer extension (step 306). In this embodiment, since the extension product is still within an intact sample cell, the extension product can be better preserved than the extension product generated with a cell/tissue extract. The method 300 thus allows an operator to perform the telomerase-mediated primer extension step immediately after receiving the sample, and store the intermediate product *i.e.*, the sample cell after the primer extension step 304, for quantification at a later time.

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needle), and other chemical or physical means to disrupt the cell membrane or improve permeability of the cell membrane. In one embodiment, the cells are suspended and are forced to pass a 25-gauge needle at least once, preferably 2-5 times. The cells are then seeded into reaction tubes or wells in a culture medium containing the first primer and dNTPs. The shearing effect of passing through the needle damages the cell membrane and allows the first primer to enter the cells. The syringe-treated cells are then incubated with the first primer and dNTPs to allow the generation of the extension product by the telomerase in the cell.

In another embodiment, the first primer and dNTPs are introduced into cells using calcium phosphate precipitation. The calcium phosphate precipitation is formed in the presence of the first primer and dNTP, and is added to the cells. The cells are then incubated at 37°C for about 10-120 min to allow the generation of the extension product by the telomerase in the cell.

The methods of the present invention may be used as diagnostic assays to determine the progression or severity of a telomerase-related disease such as Hodgkin's disease. The quantification of telomerase activity is also useful, for example, to determine the severity of the telomerase-related disease following treatment.

The detection methods described herein may be performed, for example, by utilizing prepackaged diagnostic kits containing an extension composition containing a first primer and nucleoside triphosphates, and a detection composition containing a second primer, nucleoside triphosphates, a double-stranded DNA binding dye, and a DNA polymerase. The extension composition is capable of producing an extension product from the first primer when mixed with a telomerase; and the detection composition is capable of amplifying the extension product in a PCR reaction and generating a labeled amplification product for quantification. The diagnostic kits may be conveniently used, *e.g.*, in clinical settings to diagnose subjects exhibiting symptoms or family history of a telomerase-related disease such as Hodgkin's disease. Any cell type or tissue in which telomerase is expressed may be utilized in the prognostic or diagnostic assays described herein.

In one embodiment, the telomerase activity in a biological sample is determined and an increased telomerase activity over a pre-determined normal level indicates a telomerase-related disease such as Hodgkin's disease.

The detection method described herein can also be utilized as a prognostic assay to identify subjects having or at risk of developing telomerase-related disease, such as Hodgkin's disease, that is associated with aberrant telomerase activity.

Furthermore, the prognostic assay described herein can be used to determine  
5 whether a subject can be administered a drug candidate to treat or prevent a disease associated with aberrant telomerase activity. Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disease associated with aberrant telomerase activity. Prognostic assays can be devised to determine whether a subject undergoing treatment for a telomerase-related disease has a  
10 poor outlook for long term survival or disease progression. By establishing telomerase activity profiles of different stages of the disease, from onset to later stages, an activity pattern may emerge to correlate a particular activity profile to increased likelihood of a poor prognosis. The prognosis may then be used to devise a more aggressive treatment program and enhance the likelihood of long-term survival and well-being. Similarly, the  
15 detection method of the present invention can be used in basic drug screening or clinical trials to monitor the influence of agents (*e.g.*, drugs, small molecules, proteins, nucleotides) on the activity of telomerase. For example, the effectiveness of an agent determined by a screening assay to decrease telomerase activity, can be monitored in clinical trials of subjects exhibiting increased telomerase activity. In such clinical trials,  
20 the activity of telomerase can be used as a "read-out" of the phenotype of a particular tissue.

In an embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent including the steps of (i) obtaining a pre-administration sample from the subject prior to administration of the agent; (ii)  
25 detecting the level of telomerase activity in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of telomerase activity in the post-administration samples; (v) comparing the level of telomerase activity in the pre-administration sample with the level of telomerase activity in the post-administration sample or samples; and (vi) altering the administration of the  
30 agent to the subject accordingly. For example, decreased administration of the agent may be desirable to increase the activity of telomerase to higher levels than detected, *i.e.*, to decrease the effectiveness of the agent. According to such an embodiment, telomerase activity may be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

As described herein, the telomerase detection method may be used for a variety of applications, including but are not limited to, evaluating the effectiveness of telomerase inhibitors, measuring the relationship between telomerase activity and cell culture conditions, determining the relationship between telomerase activity and aging, or  
5 between telomerase activity and tumorigenesis, diagnosing telomerase-related disease, and monitoring the treatment for such diseases.

### Examples

#### *Example 1, Determination of primer ratio*

293T cell line (primary human embryonal kidney transformed by sheared human  
10 adenovirus type 5 (Ad 5) DNA and SV 40 T-antigen, obtained from American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The cells were collected at 70-85% confluency, counted, and lysed using a lysis buffer containing 10 mM Tris-HCl, pH7.5, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM b-  
15 mercaptoethanol, 0.5% w/v CHAPS, and 10% w/v Glycerol (CHAPS lysis buffer) on ice for 30 min. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). The cell extract was stored at -70°C.

Oligonucleotide primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA). The first primer (FP) has the sequence of 5'-  
20 AATCCGTCGAGCAGAGTT-3' (SEQ ID NO:3) and is designated TS, the second primer (SP) has the sequence of 5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3' (SEQ ID NO:4) and is designated ACX. To test the effect of primer ratio of the first primer versus the second primer, different ratios (FP:SP = 1:1, 1:0.8, 1:0.5, and 1:0.2) were used. Briefly, 1 µl of cell extract at different dilutions was mixed with 1 µl of the  
25 primer mixture (0.5 µg TS/ACX at different ratio), 11.5 µl water, and 12.5 µl PCR premix buffer containing 0.25 unit DNA Polymerase, 2.5 mM of dATP, dUTP, dCTP and dGTP, and SYBR Green (1:2000 dilution of the SYBR Green 1 stock solution (S-7563) purchased from Molecular Probes Inc., Eugene, OR), incubated first at 25°C for 20 min and then at 95°C for 10 min, and amplified by quantitative real time PCR (95°C 30 sec,  
30 60°C 30 sec, 72°C 30 sec for 40 cycles). The result indicated that the ratio 1/1 (w/w) gave the best result (Table 1).

Table 1. Effect of primer ratio on quantitative real time PCR reaction

Dilutions	Cell Extract ( $\mu$ g)	FP/SP ratio	Threshold cycles ( $C_T$ )			
			1/1	1/0.8	1/0.5	1/0.2
A	0.40		20.7	25.4	28.2	27.5
B	0.08		22.5	27.0	26.9	27.9
C	0.016		25.1	27.8	28.4	30.1
D	0.0032		27.4	30.0	32.3	32.7
E	0.00064		29.6	32.0	31.2	35.4
F	0.00013		32.4	33.8	35.6	35.4
Blank	--		33.0	33.5	34.3	--

**Example 2, Determination of assay sensitivity**

To test the sensitivity of the telomerase activity assay method, the assay was performed using different amounts (or cell numbers) of 293T cell extract under the conditions described in Example 1 and a FP/SP ratio of 1/1. As shown in Table 2 and Figure 4, the method is capable of detecting telomerase activity from a single 293T cell.

Table 2. Detection of telomerase activity in 293T cells

Dilutions	Cell Extract (Per reaction)		Threshold cycles ( $C_T$ )	Numbers
	$\mu$ g	Cell		
A	2.800	8500	18.0	
B	0.82	2500	19.7	
C	0.16	500	21.1	
D	0.032	100	23.1	
E	0.0064	20	25.4	
F	0.0032	10	26.6	
G	0.0016	5	27.5	
H	0.00033	1	28.8	
Blank	--	--	33.0	

**Example 3, Generation of standard curves**

To quantify the results from real time PCR reaction, a control template molecule was used to generate a standard curve under conditions described in Example 2 to correlate telomerase activity with template molecule numbers per reaction through the threshold cycles. As shown in Table 3 and Figure 5, the standard curve was generated using the reading of the threshold ( $C_T$ ) of the real-time PCR under the standard reaction conditions and a control template TSR9. Figure 6 shows the liner regression between 293T cell number and threshold cycle and liner regression between the control template TSR9 molecule number and threshold cycle.

Table 3. Generation of the standard curve using TSR9 template control

Dilutions	TSR9 concentration		Threshold cycles ( $C_T$ )
	( $\mu\text{g}/\mu\text{l}$ )	molecules/reaction	
5			
S1	0.5	300000	18.3
S2	0.1	60000	20.8
S3	0.02	12000	22.6
S4	0.004	2400	25.2
10			
S5	0.0008	480	27.3
S6	0.00016	96	29.8
S7	0.000032	20	32.0
S8	0.0000064	4	32.5
Blank	-	-	33.0

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**Example 4, Specificity of the telomerase assay method**

To test the specificity of the telomerase assay method, the assay was performed using several tumor cell lines including MCF7 (breast pleural effusion adenocarcinoma), ZR-75-1 (breast ascites ductal carcinoma) and Hela (cervix adenocarcinoma) cells, various murine tissues (harvested from adult C57BL/6 mice) including brain, kidney, liver, heart, testis, and blood, as well as heat inactivated (95°C for 10 min) extract from 293T cells. As shown in Table 4 and Figure 7, telomerase activity is detected in proliferating cells, cancer cell lines, (293T, Hela, MCF7 and ZR-75-1), but is not detected in heat inactivated 293T cell extract and blank control. The results also indicated that there was low telomerase activity in normal adult murine tissues, indicating that the proliferating cells (e.g., stem cells) in normal tissues are detectable.

Table 4. Telomerase activity in various cell lines and murine tissues

Sample	Cell Extract (Per reaction) $\mu\text{g}$	Cycle Thresholds ( $C_T$ ) Mean $\pm$ SD
Control	--	34.3 $\pm$ 0.2
Brain	0.01	29.6 $\pm$ 0.2
35 Kidney	0.01	29.6 $\pm$ 0.2
Liver	0.01	29.4 $\pm$ 0.2
Heart	0.01	28.6 $\pm$ 0.2
Testes	0.01	28.2 $\pm$ 0.2
Blood	0.01	31.3 $\pm$ 0.1
40 MCF7	0.01	23.3 $\pm$ 0.5
ZR75	0.01	24.2 $\pm$ 0.4
Hela	0.01	24.6 $\pm$ 0.4
293T	0.01	24.3 $\pm$ 0.5
293T(heat inactivated)	0.01	34.7 $\pm$ 0.2

45

**Example 5, Stability of the reaction mixture.**

To test the stability of the reagents used in the telomerase assay, a reaction mixture containing a first primer TS, DNA Polymerase, dNTPs and SYBR Green was prepared. Aliquots of the reaction mixture were kept at room temperature for 0, 24, 48 or 72 h, and tested in a telomerase assay. As shown in Table 5, the reaction mixture seems to be stable at room temperature for up to at least 72 hours.

Table 5. Stability of the reaction mixture at room temperature

Dilutions	Cell Extract ( $\mu$ g)	Exposure at RT (hrs)	Threshold cycles ( $C_T$ )			
			0	24	48	72
A	2.0		18.0	16.7	18.2	16.4
B	0.40		21.6	22.4	21.9	20.9
C	0.08		22.3	21.8	21.9	21.4
D	0.016		24.3	24.2	23.9	24.2
F	0.0032		26.1	25.7	25.6	25.2
G	0.00064		27.9	27.7	28.2	27.6
H	0.00013		28.3	28.4	28.6	31.5
Blank	--		34.3	32.1	33.9	
	32.9					

**Example 6, Primer extension in intact cells**

293T cells were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO<sub>2</sub>). The cells were trypsinized, washed with the growth medium, suspended in the growth medium at a density of 1 x 10<sup>6</sup> cells/ml, and subjected to one of the following treatment:

**Treatment A:** The cell suspension was transferred to a sterile centrifuge tube, centrifuged at 800 rpm for 5 min in a Beckman GS-6R centrifuge, re-suspended in phosphate buffered saline (PBS). The re-suspended cells were drawn up in a sterile 1-ml syringe through a 25-gauge needle and then expelled by steady pressure on the plunger. The syringe procedure was repeated five times. The cells were transferred into sterile centrifuge tubes at 1 x 10<sup>4</sup> cells / tube and a transfer medium (2.5 mM dNTP and 0.1% bovine serum albumin (BSA)) was added, either with or without TS primer. The cells were cultured at 37° C for 60 min. The tubes were centrifuged at 4°C for 20 min. and suspensions were collected. The pellets were lysated using Chaps buffer at 4° C for 30



min. The lysates were centrifuged at 14,000 rpm for 20 min at 4°C. The supernatants were collected, heated at 95°C for 10 min, and stored at – 70°C before use.

Alternatively, the cells may be seeded in 96 well plates at a density of  $1 \times 10^4$  cells/well after the syringe procedure and cultured overnight at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The next day, the cells may be washed with PBS and a desired amount of transfer buffer (PBS with TS primer, 0.5 µg/well, dNTP 2.5mM and 0.1% BSA; or PBS with dNTP, 2.5mM and 0.1%BSA, or PBS only) is added to each well. After incubation at 37°C for 30 min, 60 min, and overnight, the cells are collected and lysated with the CHAPS buffer at 4°C for 30 min. The lysates are centrifuged at 14,000 rpm. The supernatants are collected, heated at 95°C for 10 min, and stored at – 70°C before use.

*Treatment B:* The cell suspension was directly seeded in a 96-well plate, cultured overnight, and transfected with a desired amount of TS primer and dNTP using calcium phosphate precipitation. Briefly, the 293T cells grew in growth medium (DMEM with 10%

FBS) overnight. The growth medium was replaced with fresh growth medium 3 hours before the transfection. Calcium phosphate precipitation (using the Calcium phosphate kit from GIBCO) was formed in the presence of the TS primer and dNTP (HBS 100 ul, phosphate 2 ul, H<sub>2</sub>O 26 ul, carrier DNA 10 ul, first primer 20 ul, 0.25 mM dNTP, 12 ul calcium) and added to the cells. The cells were incubated at 37°C for 10, 30, and 60 min, and were lysed with CHAPS lysis buffer at 4°C for 30 min. The lysates were heated at 100°C for 10 min and centrifuged at 14,000 rpm for 20 min. The supernatants were collected and stored at -70°C.

One microliter of the lysate from treatment A or B was mixed with 11.5 µl water and 12.5 µl premix containing dNTPs (2.5 mM for each nucleoside triphosphate), 0.25 unit of DNA polymerase, 0.25 µg ACX primer, and subjected to quantitative real-time PCR ( 95°C for 10 min, 95°C 30 sec, 60°C 30 sec, 72°C 30 sec for 35 – 40 cycles). The results shown in Figure 8 indicate that telomerase activity is detectable in 293T cells after an one hour incubation with the TS primer and dNTP as described in treatment A. The results shown in Figure 9 indicate that telomerase activity is detectable in 293T cells after a 30-minute incubation with calcium phosphate precipitate containing the TS primer and dNTP, as described in treatment B. No telomerase activity was detected without the TS primer, suggesting that the products detected are telomerase specific.

Although preferred embodiments and their advantages have been described in detail, various changes, substitutions and alterations can be made herein without departing from the scope of the compositions and methods as defined by the appended claims and their equivalents and all such are intended to be within the scope of the

5 appended claims.